ปริมาณวิเคราะห์สารเควอซีติน และเควอซิตริน ในใบของพืชสกุลชงโค ที่พบในประเทศไทยโดยวิธี RP-HPLC

RP-HPLC Preliminary Analysis of Quercetin and Quercitrin Contents in *Bauhinia* spp. Leaves Distributed in Thailand

นิพนธ์ต้นฉบับ

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Original Article

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บทคัดย่อ

วัตถุประสงค์: การศึกษาเบื้องต้นเพื่อหาปริมาณสารเควอซีติน และเควอซิตรินในใบของพืชสกุลชงโค 20 สายพันธุ์ในประเทศไทย **วิธีการศึกษา:** เก็บใบเพสลาดของพืชสกุลชงโคทั้ง 20 สายพันธุ์ นำมาทำความสะอาด อบแห้ง และสกัดด้วยเอทานอล (ร้อยละ 95) โดยการสกัดแบบต่อเนื่อง แยกสารโดยวิธีโครมาโทกราฟีของเหลวสมรรถนะสูง ที่อุณหภูมิ 35 องศาเซลเซียส โดยใช้คอลัมน์ Inersil® ODS-3 C_{18} เป็นเฟสคงที่ และใช้สารละลายของกรดฟอสฟอริก (ร้อยละ 0.5) กับเมทานอล ในอัตราส่วน 1 ต่อ 1 เป็นเฟสเคลื่อนที่ ตรวจวัดปริมาณเควอซีตินและเควอซิตรินด้วยดีเทคเตอร์ชนิดโฟโต้ไดโอดอาเรย์ที่ 255 นาโนเมตร **ผลการศึกษา:** สัมเสี้ยวเถาให้ปริมาณสิ่งสกัดมากที่สุด (36.13 กรัมต่อ 100 กรัมโดยน้ำหนักแห้ง) และเสี้ยวดอกขาวให้น้อยที่สุดใน (16.06 กรัมต่อ 100 กรัมโดยน้ำหนักแห้ง) กาหลง ใบไม้สีทอง กาหลงดอกแดง เถาไฟ สัมเสี้ยวเถา สัมเสี้ยวปอเกี๋ยน ชงโคดำ ชงโค เถากระไดลิง สร้อยสยาม สิรินธรวัลลี เถาขยัน และคิ้วนาง พบทั้งสารเควอซีติน และเควอซิตริน สัมเสี้ยวพบสารเควอซีติน และเควอซิตรินมากที่สุดเท่ากับ 191.81 and 373.97 มิลลิกรัมต่อ 100 กรัมโดยน้ำหนักแห้ง สารเควอซีตินไม่พบในแสลงพัน ชงโคนา เสี้ยวป่า และโยทะกา ส่วนสารเควอซิตรินไม่พบในแสลงพันเถา และเสี้ยวดอกขาว การตรวจสอบความใช้ได้ของวิธีวิเคราะห์ได้ถูกทดสอบเพื่อยืนยันความแม่นยำ และถูกต้องของวิธีวิเคราะห์ **สรุป:** วิธีโครมาโทกราฟีของเหลวสมรรถนะสูงโดยดีเทคเตอร์ชนิดโฟโต้ไดโอดอาเรย์มีประสิทธิภาพดีในการแยกและวิเคราะห์ปริมาณสารเควอซีติน และเควอซิตรินในพืชสกุลชงโคทั้ง 20 สายพันธุ์

คำสำคัญ: สกุลชงโค, เควอซีติน, เควอซิตริน, วิธีโครมาโทกราฟีของเหลวสมรรถนะสูง

Abstract

Objective: To preliminarily quantitate quercetin and quercitrin in mature leaves of *Bauhinia* species distributed throughout Thailand using RP-HPLC analysis. **Methods:** Mature leaves of 20 *Bauhinia* species were collected, cleaned and exhaustively extracted with 95% ethanol using Soxhlet apparatus. The ethanolic extracts were injected to Inertsil® ODS-3 C_{18} column at 35 ºC. The elution solvent was 0.5% phosphoric acid:methanol (1:1) at the flow rate of 1.0 ml/min. Photo-diode array detector was set at 255 nm. **Results:** The highest yield was found in *B. lakhonensis* (36.13 g/100 g dried leaves) and the lowest yield in *B. variegata* (16.06 g/100 g dried leaves). *B. acuminata*, *B. aureifolia*, *B. galpinii*, *B. integrifolia*, *B. lakhonensis*, *B. malabarica*, *B, ornata*, *B. pottsii*, *B. purpurea*, *B. scandens*, *B. siamensis*, *B. sirindhorniae*, *B. strychnifolia* and *B. winitii* were found to have both quercetin and quercitrin. The highest contents of quercetin and quercitrin were found in *B. malabarica* as 191.81 and 373.97 mg/100 g dried leaves, respectively. Quercetin was not found in *B. pulla*, *B. racemosa*, *B. saccocalyx*, and *B. tomentosa*. Quercitrin was not found in *B. bracteata*, and *B. variegata*. The validity of the analysis was in the acceptable range. **Conclusion:** RP-HPLC with PDA detector performed a good separation and could quantitate quercetin and quercitrin content in selected 20 *Bauhinia* species distributed throughout Thailand.

Keywords: *Bauhinia* spp., quercetin, quercitrin, RP-HPLC

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Introduction

Bauhinia, a large genus of the family Leguminosae, consists of 300 species of trees, shrubs and climbers which are normally known as 'cow's paw' or 'cow's hoof' because of their leaves shape. They are widely distributed in most warm countries. In India, the bark of *B. purpurea* and *B. variegata* is astringent so it is used for astringent poultice. The buds of *B. variegata* are useful for diarrhea.[1] Some other species are used to treat cough. In the Philippines, the bark of *B. tomentosa* and *B. malabarica* are used against dysentery. In Thailand, *B. malabarica* has been used in traditional medicine for treating many diseases, e.g. headache, fever and urinary disorder.[2]

The main chemical compounds in plants of *Bauhinia* are usually encountered flavonoids especially kaempferol and quercetin derivatives.[3-5] Flavonoid is an essential class of plant secondary metabolites normally found in several parts of the plant as water soluble glycosides in the vacuoles of the epidermal cells.[6,7] They are the key of plant growth, plaques

protection[8] and most of them are recognized as pigments of flowers in the angiosperm families.[7] Quercetin is an aglycone flavonoid, and quercetin that binds to rhamnose is called quercitrin (quercetin-3-O-rhamnoside) (Figure 1). Quercetin has diverse pharmacological activities, for example improving blood circulation, lowering blood pressure, anti-inflammatory, anti-allergy, antimicrobial, and antitumor activities.[9-11] In addition, quercitrin also has UV protection, antitumor, antimicrobial, anti-aging and anti-allergy activities.[12-14] Additionally, both quercetin and quercitrin were reported that they had strong antioxidant activity in many studies.[2,15,16]

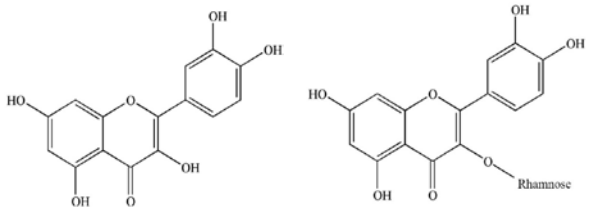

Figure 1 Structures of quercetin (left) and quercitrin (right).

There are more than 40 *Bauhinia* species distributed throughout Thailand.[17] However, studies of them are rarely explored and still lack of chemical quantification especially quercetin and its glycoside. The aim of this study was to establish quercetin and quercitrin contents in 20 *Bauhinia* species throughout Thailand using RP-HPLC analysis.

Methods

Sample collection

The mature leaves of selected *Bauhinia* species were collected throughout Thailand (January 2016 – July 2018) and dried at 45 ºC in hot air oven. All plants materials were authenticated by one of the authors (N. R.) and herbarium comparison at the Forest Herbarium-BKF. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University. Crude drugs were pulverized after removal of any foreign matters.

Twenty *Bauhinia* species used in this study were *Bauhinia acuminata*, *B. aurefolia*, *B. bracteata*, *B. galpinii*, *B. integrifolia*, *B. lakhonensis*, *B. malabarica*, *B. ornata*, *B. pottsii*, *B. pulla*, *B. purpurea*, *B. racemosa*, *B. saccocalyx*, *B. siamensis*, *B. scandens*, *B. sirindhorniae*, *B. strychnifolia*, *B. tomentosa*, *B. variegata*, and *B. winitii* (Table 1).

Sample extraction

Five grams of each dried leaf powder of twenty *Bauhinia* species were exhaustively extracted with 95% ethanol (300 ml) using a Soxhlet apparatus until the solvent remaining in the thimble was clear (around 48 hours). The ethanolic extract was filtered through filter-paper Whatman No. 4 and evaporated to dryness in vacuo. The extracted yields were recorded and stored at -20 ºC to avoid the possibility of degradation of active compounds.

Table 1 Twenty *Bauhinia* species in three different collecting locations.

No.	Species	Locality		
1	*Bauhinia acuminata*	Bangkok 1	Bangkok 2	Chiang Rai
2	*B. aureifolia*	Bangkok 1	Bangkok 2	Trang
3	*B. bracteata*	Bangkok 1	Bangkok 2	Kanchanaburi
4	*B. galpinii*	Bangkok	Trang	Chiang Rai
5	*B. integrifolia*	Nonthaburi	Bangkok	Chiang Rai
6	*B. lakhonensis*	Bangkok	Chiang Rai	Nong Khai
7	*B. malabarica*	Bangkok	Chonburi	Pathum Thani
8	*B. ornata*	Chiang Rai	Lampang	Kanchanaburi
9	*B. pottsii*	Bangkok	Chiang Rai	Satun
10	*B. pulla*	Nakhon Sawan	Singburi	Chai Nat
11	*B. purpurea*	Bangkok	Chiang Rai	Lampang
12	*B. racemosa*	Bangkok 1	Bangkok 2	Singburi
13	*B. saccocalyx*	Bangkok	Ratchaburi	Rayong
14	*B. scandens*	Bangkok	Nonthaburi	Kanchanaburi
15	*B. siamensis*	Phitsanulok 1	Phitsanulok 2	Phitsanulok 3
16	*B. sirindhorniae*	Bangkok	Nonthaburi	Kanchanaburi
17	*B. strychnifolia*	Bangkok	Pathum Thani	Chiang Rai
18	*B. tomentosa*	Bangkok 1	Bangkok 2	Pathum Thani
19	*B. variegata*	Lampang 1	Lampang 2	Lampang 3
20	*B. winitii*	Bangkok 1	Bangkok 2	Kanchanaburi

Chromatographic condition

Shimadzu HPLC LC-20A system (Shimadzu, Japan) consisted of system controller (CMB-20A), two solvent delivery units (LC-20A), an on-line degassing unit (DGU-20A3), an auto-sample (SIL-20A), a column oven (CTO-20A) and a photo-diode array detector (SPD-M20A). System control and data analysis were processed with Shimadzu LC Solution software. The chromatographic condition was developed and recently published elseware[18], i.e. the solvent system was set as isocratic elution mode with 50% of methanol and 50% of 0.5% v/v phosphoric acid in water (total run time of 30 minutes) and analyzed using Inertsil® ODS-3 5µm C_{18} column (4.6x250 mm) coupled with ReproSil®-Pur ODS-3 C_{18} guard column (4.0x10 mm). Flow rate was 1.0 ml/min, and column temperature was 35 ºC. The extracts and standards (quercetin and quercitrin from ChromaDex, California, United States) were dissolved in methanol, filtered through 0.45 µm PTFE membrane syringe filter and injected volume was 5 µl. Peak

areas were observed under 255 nm and calculated using linear equations from calibration range of quercetin and quercitrin (20, 40, 60, 80 and 100 µg/ml). Method validation was performed according to the ICH guideline.[19]

Results and Discussions

This RP-HPLC condition was suitable for the separation of quercetin and quercitrin in ethanolic leaf extracts of selected twenty *Bauhinia* species. The method validity for quantitative analysis was performed on *B. malabarica* and recently published elsewhere.[18] The analytical performance characteristics are shown in Table 2. The highest contents of quercetin and quercitrin were found in *B. malabarica* as 191.81 and 373.97 mg/100 g of dried leaves respectively. Quercetin was not found in *B. pulla*, *B. racemosa*, *B. saccocalyx*, and *B. tomentosa*. Quercitrin was not found in *B. bracteata*, and *B. variegata* (Table 3).

Table 2 Validity of quercetin and quercitrin quantification.

Parameter	Quercetin	Quercitrin
Calibration range	y = 18199x – 31136	y = 14702x – 6863.3
Accuracy (% recovery) #	97.39, 97.38, 99.18	98.61, 98.18, 102.29
Repeatability (% RSD) #	1.15, 1.50, 1.16	1.42, 1.55, 1.43
Intermediate precision (% RSD)#	2.95, 2.52, 1.52	0.81, 2.95, 1.13
Limit of detection (µg/ml)	4.76	1.94
Limit of quantification (µg/ml)	14.41	5.88
Robustness (% RSD) ##		
Flowrate 0.950 - 1.050 ml/min	4.05, 6.78	4.01, 7.04
Column temperature 34 - 36 °C	3.03, 6.85	2.60, 6.64
Wavelength 252 - 258 nm	0.09, 2.55	0.13, 1.86

Low, medium, high concentration; ## Retention time, peak area

Table 3. Quercetin and quercitrin contents in selected twenty *Bauhinia* species.

No.	Scientific plant name	Yield of the extract (g/100 g dried leaf)	Quercetin (mg/100 g dried leaf)*	Quercitrin (mg/100 g dried leaf)*
1	*Bauhinia acuminata* L.	26.13	20.67 ± 0.18	38.70 ± 0.02
2	*B. aureifolia* K.&S.S.Larsen	18.76	64.11 ± 0.06	96.76 ± 0.24
3	*B. bracteata* (Graham ex Benth.) Baker	17.19	11.98 ± 0.02	-
4	*B. galpinii* N.E.Br.	23.90	40.05 ± 0.04	11.90 ± 0.04
5	*B. integrifolia* Roxb.	24.50	8.37 ± 0.08	359.64 ± 0.98
6	*B. lakhonensis* Gagnep.	36.13	139.03 ± 0.36	321.64 ± 1.42
7	*B. malabarica* Roxb.	26.20	191.81 ± 0.61	373.97 ± 0.24
8	*B. ornata* Kurz	17.53	65.97 ± 0.01	46.16± 0.14
9	*B. pottsii* G.Don	19.16	16.27 ± 0.03	206.20 ± 0.52
10	*B. pulla* Craib	30.04	-	94.24 ± 0.32
11	*B. purpurea* L.	17.15	2.66 ± 0.01	19.28 ± 0.51
12	*B. racemosa* Lam.	21.55	-	15.69 ± 0.10
13	*B. saccocalyx* Pierre	23.79	-	211.52 ± 0.05
14	*B. scandens* L.	19.54	4.20 ± 0.01	51.38 ± 0.48
15	*B. siamensis* K.&S.S.Larsen	21.15	6.25 ± 0.02	350.36 ± 0.44
16	*B. sirindhorniae* K.&S.S.Larsen	25.52	13.25 ± 0.10	148.35 ± 0.23
17	*B. strychnifolia* Craib	28.38	31.17 ± 0.02	45.47 ± 0.22
18	*B. tomentosa* L.	24.25	-	24.43 ± 0.08
19	*B. variegata* L.	16.06	4.74 ± 0.01	-
20	*B. winitii* Craib	30.66	41.59 ± 0.07	45.47 ± 0.05

* Mean ± SD of triplicate HPLC analysis.

RP-HPLC is the popular method which is used for the separation of secondary metabolites in plants. In this study, RP-HPLC exhibited a potential in separating quercetin and quercitrin in all 20 *Bauhinia* species. *B. malabarica* leaves have been used in Thai traditional remedy for a long time. Seven flavonols including quercetin and quercitrin have been isolated from the methanolic extracts of *B. malabarica* leaves by various chromatographic techniques.[2] In this study, the ethanolic leaf extracts of 14 *Bauhinia* species contained both quercetin and quercitrin. *B. malabarica* leaves contained the highest amounts of quercetin and quercitrin. *B. bracteata* and *B. variegata* leaves contained only quercetin. The study in Brazil also reported only quercetin found in 70% ethanolic leaf extracts of *B. variegata*.[20] However, the content of quercetin and quercitrin in *Bauhinia* species in this study was preliminary because only one sample of each species was used for quantification.

In this study, the optimum wavelength was set at 255 nm which could be absorbed both by quercetin and quercitrin. Peak purity determination based on selected multiple spectral inputs of diode array detector is capable to differentiate co-eluted compounds. If the peak is pure, spectra taken at several points during a peak elution should all be identical.[21] Method validation is done to confirm the reliability of the quantitative analysis. In this study, the quantification of quercetin and quercitrin in *Bauhinia* leaf extracts were developed. The results of method validation of this study were in the acceptable range. The acceptable % recovery is between 80 - 120%.[18] The result of % RSD determined the error of the method, where the acceptable RSD was not more than 15%.[22] The small variations of column temperature, flow rate and detection wavelength resulted in % RSD < 8, so the method was robust.

Conclusion

The reversed phase HPLC with PDA detector performed the good separation and could quantitate quercetin and quercitrin content in 20 selected *Bauhinia* species distribing in Thailand. Quercetin and quercitrin could be used as the chemical markers in *Bauhinia* species. Further studies to specify the amounts of these markers could be performed by collecting the leaf samples from 12 - 15 locations per species.

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